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used alone, the universality of the method together with its high cell type selectivity can easily be achieved by the use of various targeting conjugates. The development of a Moloney murine leukemia virus with an Fc.gamma.RI Ig-binding domain on the viral surface is described.

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AB Skeletal muscle is an attractive target for somatic gene transfer of both acquired and inherited disorders. Direct injection of adenoviral vectors in the skeletal muscle leads to recombinant gene expression in a large no. of muscle fibers. Transgene expression has been transient in most organs and assocd. with substantial inflammation when expts. are performed in adult immune competent mice. In this report, we utilize a variety of in vivo and in vitro models of T and B cell function to characterize the nature of the immune response to adenoviral vectors injected into murine skeletal muscle. Cellular immunity dependent on CD4+ and CD8+ T cells contributes to the loss of recombinant gene expression and the development of localized inflammation. Antigen

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# Immunology of gene therapy with adenoviral vectors in mouse skeletal muscle

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Skeletal muscle is an attractive target for somatic gene transfer of both acquired and inherited disorders. Direct injection of adenoviral vectors in the skeletal muscle leads to recombinant gene expression in a large number of muscle fibers. Transgene expression has been transient in most organs and associated with substantial inflammation when experiments are performed in adult immune competent mice. In this report, we utilize a variety of *in vivo* and *in vitro* models of T and B cell function to characterize the nature of the immune response to adenoviral vectors injected into murine skeletal muscle. Cellular immunity dependent on CD4<sup>+</sup> and CD8<sup>+</sup> T cells contributes to the loss of recombinant gene expression and the development of localized inflammation. Antigen specific activation of T cells occurs to both viral proteins and the reporter gene  $\beta$ -galactosidase. Systemic levels of neutralizing antibody to the capsid proteins of the vector are also generated. Destructive immune responses responsible for loss of transgene expression are largely directed against  $\beta$ -galactosidase in that transgene expression was stable when  $\beta$ -galactosidase was eliminated as a neoantigen in mice transgenic for *lacZ*. A strategy to prevent the cellular and humoral immunity to this therapy was developed based on transiently ablating CD4<sup>+</sup> T cell activation at the time of vector delivery. Encouraging results were obtained when vector was administered with one of several immune modulating agents including cyclophosphamide, mAb to CD4<sup>+</sup> cells, and mAb to CD40 ligand. These studies indicate that cellular and humoral immune responses are elicited in the context of gene therapy directed to skeletal muscle with adenoviral vectors. Transient

ablation of CD4<sup>+</sup> T cell activation prevents the effector responses of the CD8<sup>+</sup> T and B cells.

## INTRODUCTION

Recombinant adenoviruses are being tested as vehicles to deliver genes to skeletal muscle. Applications include treatment of inherited neuromuscular diseases such as Duchenne Muscular Dystrophy (DMD) (1), as well as ectopic expression of a gene whose product is secreted in the blood stream (2,3). First generation adenoviral vectors have been deleted of sequences spanning the immediate early genes E1a and E1b to render them replication defective and potentially suitable for therapeutic applications. The fact that adenoviral vectors can efficiently transduce nondividing cells is important for *in vivo* gene transfer to skeletal muscle where the majority of cells are syncytium of post-mitotic cells called muscle fibers. Injection of vector into newborn mice yields efficient and stable recombinant gene expression which contrasts with the results obtained in adult animals where expression of the transgene diminishes to undetectable levels within several weeks (4-10).

The observation that adenoviral vector expression is prolonged when administered to a newborn suggested that host responses may underlie limitations of the technology in adults. This is reminiscent of previous studies with adenoviral vectors delivered to mouse liver and lung where a full spectrum of humoral and cellular immune responses have been characterized (11-14). In these systems, CD8<sup>+</sup> T cells are activated to neoantigens expressed in the target cells which include viral proteins and the transgene product. Resulting cytotoxic T lymphocytes (CTLs) target the genetically modified cell leading to extinction of transgene expression and the development of inflammation. The input viral capsid proteins activate B cells that secrete neutralizing antibody which diminishes efficiency of gene transfer upon a second administration. *In vivo* activity of the CTL and B cell effectors is dependent on activation of CD4<sup>+</sup> T cells to the viral antigens (15-17). Confirmation of the role of cellular and

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humoral immune responses in muscle directed gene transfer has been supported in previous studies in immune deficient or immune suppressed animals where transgene expression was extended and inflammation was diminished (8–10,18).

In this report we characterize the precise cellular and humoral immune responses to E1 deleted adenoviral vector in skeletal muscle of mice. Strategies for circumventing these problematic responses are also described.

## RESULTS

### Cellular immune responses limit adenoviral vectors in skeletal muscle

An E1 deleted adenoviral vector expressing  $\beta$ -galactosidase was injected directly into the tibialis anterior muscle of C57BL/6 mice. Representative micrographs of X-gal stained tissues are presented in Figure 1, while Table 1 summarizes quantitative morphometric analyses. Evaluation of tissue 10 days later revealed high levels of  $\beta$ -galactosidase in muscle fibers located in proximity to the area injected (Fig. 1A). Expression was transient diminishing to undetectable levels within 30 days (Fig. 1B shows tissue harvested at day 60). A significant inflammatory response developed subsequent to gene transfer that was characterized by mononuclear cell infiltration and degeneration of muscle fibers (Fig. 2); inflammation peaked 17 days following gene transfer and slowly diminished to baseline over several months. No inflammation was noted in PBS injected animals (data not shown). Immunofluorescent studies indicated that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells were mobilized in this inflammatory response. Figure 3A and B show representative micrographs before and 10 days after gene transfer; Figure 3F presents a quantitative morphometric analysis of density of CD4<sup>+</sup> T and CD8<sup>+</sup> T cells 10 days after vector administration. Those cells surrounded by CD4<sup>+</sup> T and CD8<sup>+</sup> T cells were shown in serial sections to express  $\beta$ -galactosidase (data not shown).

**Table 1.** Adenovirus-mediated *lacZ* gene expression in muscles of different strains of adult mice

	Percent transgene expression		
	Day 10	Day 60	Day 180
C57BL/6	35.20 $\pm$ 14.21	0	
Rag-2 <sup>-</sup>	6.6 $\pm$ 4.6	18.03 $\pm$ 13.67	23.81 $\pm$ 5.61
$\beta$ 2m <sup>-</sup>	25.20 $\pm$ 21.78	27.31 $\pm$ 22.61	29.47 $\pm$ 19.01
Rosa-26	16.42 $\pm$ 8.84	9.13 $\pm$ 5.67	25.35 $\pm$ 9.73

Data were quantified with Quantimet 500+ (Leica) by analyzing the whole cross sections of tibialis anterior muscles from a total of six legs for the expression of *lacZ* at days 10, 60 and 180. Percent transgene expression represents the number of X-gal positive fibers divided by the total number of fibers  $\times$  100. Data represent mean  $\pm$  SD.

The role of cellular immune responses was confirmed in studies performed with mice genetically deficient in several genes involved in immune regulation. Transgene expression was stable over 180 days (Fig. 1C, D and E and Table 1) and associated with little inflammation when injected into Rag-2<sup>-</sup> mice who have global defects in both cellular and humoral immunity due to a deficiency in the recombination gene. Similar results were obtained

in  $\beta$ 2m<sup>-</sup> mice which are deficient in expression of class I MHC and lack mature CD8<sup>+</sup> T cells (Fig. 1F, G and H and Table 1).

### $\beta$ -galactosidase is the target of destructive cellular responses

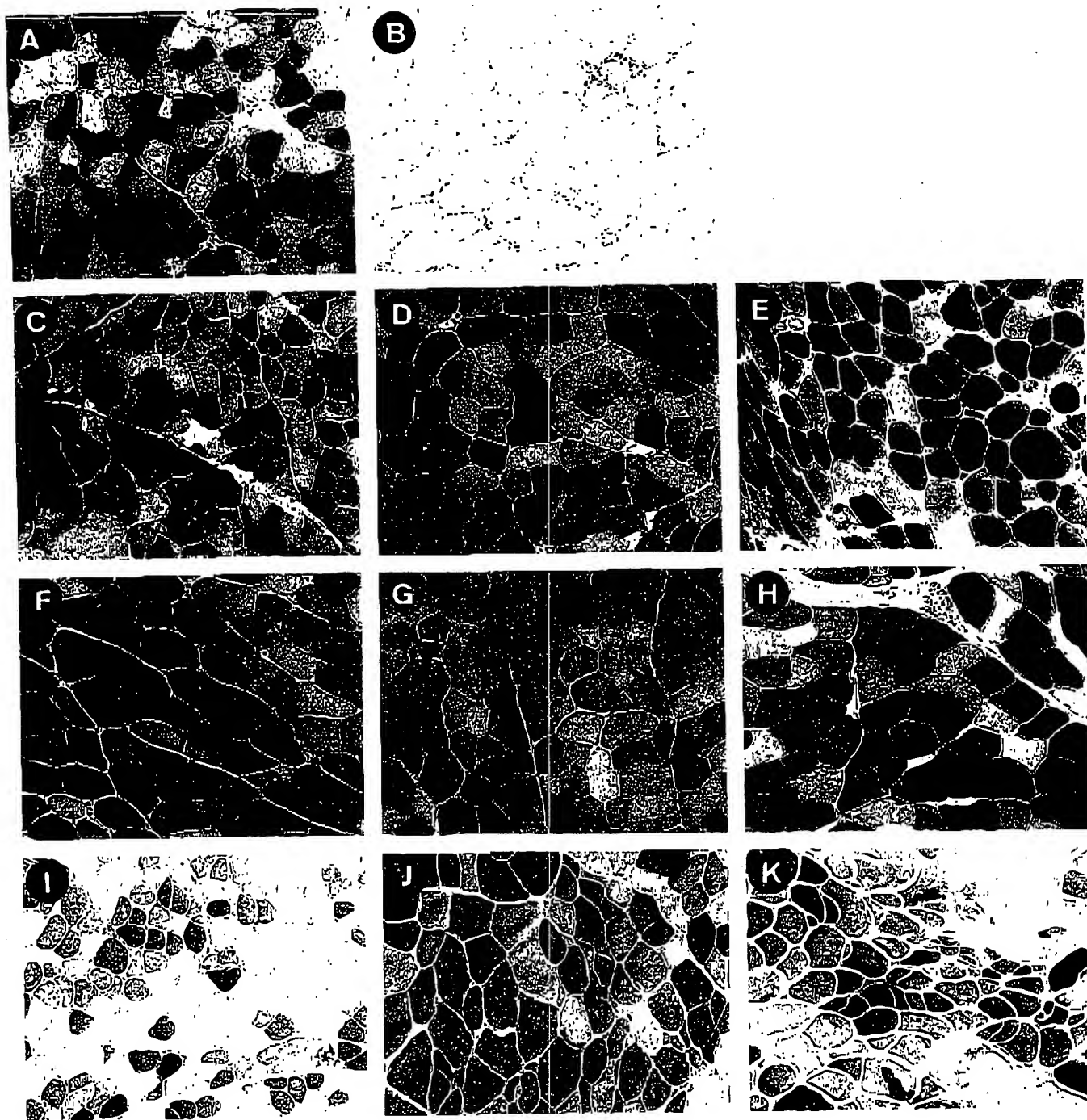
Lymphocytes were harvested from inguinal lymph nodes 10 days after gene transfer for more detailed immunologic studies including chromium release assays to assess activation of CD8<sup>+</sup> T cells and formation of CTLs. Representative experiments are presented in Figure 4. Analysis of lymphocytes harvested from C57BL/6 animals infected with H5.010CMVlacZ is shown in Figure 4A. Lymphocytes restimulated with *lacZ* expressing virus demonstrated significant cytotoxicity to MHC identical targets infected with the *lacZ* adenovirus. The lack of cytotoxic activity to uninfected targets confirmed the specificity of the assay. This experiment demonstrates CTL to vector encoded antigens but does not discriminate between viral proteins versus the expressed transgene product as the source of T cell activation. Similar results were obtained with targets infected with an adenoviral vector expressing another reporter gene indicating that viral proteins are recognized (data not shown). Cytotoxicity of targets expressing  $\beta$ -galactosidase from a retrovirus confirmed that the transgene product is also a target for CTLs.

C57BL/6 mice were injected intramuscularly (i.m.) with an identical dose of H5.010CMVlacZ that had been inactivated with UV. The goal of this experiment was to discriminate between capsid viral antigens versus newly expressed viral proteins as activators of CTLs. Lymphocytes harvested from these animals were restimulated *in vitro* and analyzed for CTL activity (Fig. 4C). No cytotoxicity above background was detected from targets infected with the adenoviral vector confirming that capsid proteins are weak activators of CTLs.

Studies described above indicate that cellular immune responses limit duration of vector derived transgene expression and that CTLs are activated to both viral antigen and  $\beta$ -galactosidase. Additional studies were performed to assess the contribution these different sets of CTLs play in extinguishing transgene expression. The *lacZ* vector was injected i.m. into the ROSA-26 mouse which is transgenic for *lacZ* expressed from a retrovirus; the germline *lacZ* gene is expressed in many tissues except for muscle. In this transgenic mouse,  $\beta$ -galactosidase should not be immunogenic, allowing a direct assessment of the performance of the vector. Lymphocytes from these animals were shown to demonstrate significant cytotoxic activity to viral protein expressing targets but not  $\beta$ -galactosidase expressing targets (Fig. 4B). The vector derived  $\beta$ -galactosidase was detectable in muscle of the ROSA-26 without diminution for at least 180 days (Fig. 1I–K). These data indicate that cellular immune responses to the vector or viral gene products do not limit duration of transgene expression. This confirms the recent report of Tripathy *et al.* who studied the performance of adenoviral vectors expressing either human or mouse erythropoietin in immune competent mice (19).

### Transient ablation of CD4 activation prevents destructive cellular immune responses

Previous studies in liver and lung models of gene therapy indicated that activation of CD4<sup>+</sup> T cells to input viral capsid proteins was necessary for functional CTL response (15–17,20,21). This would predict that administration of agents

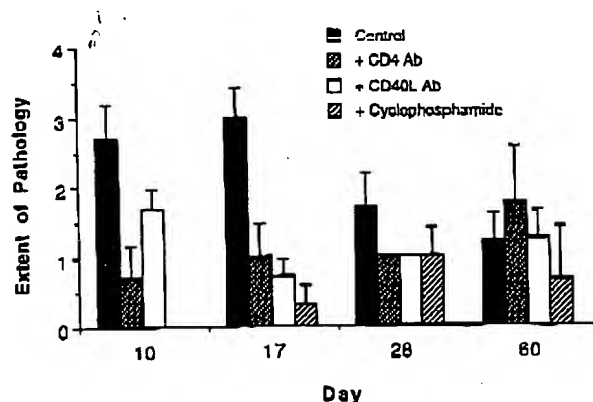


**Figure 1.** Adenovirus-mediated *lacZ* gene expression in mouse muscles. Suspensions of adenovirus H5.010CMVlacZ ( $5 \times 10^8$  p.f.u.) were injected i.m. into the tibialis anterior muscles of mice which were subsequently euthanized, and the muscle tissues were evaluated for *lacZ* expression by X-gal histochemistry 10 (panels A, C, F and I) and 60 (panels B, G and J) and 180 (panels E, H, and K) days later. (A) and (B), C57BL/6 mice; (C), (D) and (E), RAG-2<sup>-</sup> mice; (F), (G) and (H),  $\beta_2m^{-}$  mice; (I), (J) and (K), ROSA-26 mice. Magnification  $\times 200$ .

that interfere with T cell priming for a short interval after vector delivery should lead to prolongation of transgene expression. Experiments were conducted in C57BL/6 mice that received *lacZ* vector i.m. with one of the following immune modulating agents: (i) cyclophosphamide [100 mg/kg intraperitoneally (i.p.) days 0, +3 and +6], an alkylating agent that non-specifically kills cycling cells; (ii) antibody to CD4<sup>+</sup> (100  $\mu$ g day -1, 0, +3 and +6) which

specifically eliminates CD4<sup>+</sup> T cell function by depleting the cells; and (iii) blocking antibody to CD40 ligand (CD40L) (100  $\mu$ g days -1, 0, +3 and +6) that interferes with a costimulatory step required for T cell activation (22).

Lymphocytes harvested from inguinal lymph nodes 10 days after treatment with vector were stimulated with inactivated virus and supernatants were analyzed by ELISA for cytokines specific



**Figure 2.** Histopathology of mouse muscles in response to recombinant adenoviruses. Muscle tissues were harvested following infusion of H5.010CMVlacZ ( $5 \times 10^6$  p.f.u.) and evaluated for evidence of pathological changes by light microscopic examination of H&E sections. Extent of pathology was scored from 0 (no pathology) to 3 (severe pathology). See Materials and Methods for details. This figure summarizes the extent of pathology observed in C57BL/6 ('Control'), CD4 Ab-treated ('+CD4 Ab'), CD40L Ab-treated ('+CD40L Ab') and cyclophosphamide-treated ('+Cyclophosphamide') mice as a function of time following infusion of virus (days 10, 17, 28 and 60).

for either  $T_H1$  (i.e., IL-2 and IFN- $\gamma$ ) or  $T_H2$  (i.e., IL-4 and IL-10) subsets of T helper cells (Fig. 5). Lymphocytes from animals that received vector but not immune modulator secreted both  $T_H1$  and  $T_H2$  specific cytokines in response to viral antigen. Cyclophosphamide, CD4 mAb and CD40L mAb all suppressed the activation of both  $T_H$  subsets with the most substantial and consistent effect achieved with cyclophosphamide.

Muscle tissue was harvested following treatment with vectors and immune modulator and analyzed for inflammation (Fig. 2), CD4/CD8 infiltration (Fig. 3), and  $\beta$ -galactosidase expression (Figure 6 presents representative micrographs of X-gal stained tissues while Table 2 summarizes quantitative morphometric analyses). The most impressive effect was achieved with cyclophosphamide. Inflammation and infiltration of CD4/CD8 cells were eliminated at day 10 and suppressed at subsequent times; transgene expression was stabilized without apparent diminution for at least 60 days (Fig. 6J-L). Qualitatively similar effects were achieved with the CD4 and CD40L mAbs. In each case, inflammation was diminished, as were CD4 and CD8 infiltrates (CD4 lymphocytes were not observed in animals treated with CD4 mAb); transgene expression diminished 3-fold following both treatments but was still detectable at day 60 (Fig. 6D-F, CD4 mAb; Fig. 6G-I, CD40L mAb).

#### Humoral immunity to adenoviral vectors delivered to skeletal muscle

Animals treated with vector developed substantial quantities of neutralizing antibody to virus within 3 weeks (Fig. 7). The absolute quantity of neutralizing antibody was quite variable between different animals, however, it was always detected. This antiviral antibody diminishes but does not block gene transfer following a second administration of vector (data not shown). Treatment with cyclophosphamide, CD4 mAb or CD40L mAb completely suppressed the formation of neutralizing antibody (Fig. 7).

**Table 2.** The effect of immune modulators on the stability of transgene expression in mouse skeletal muscles

	Percent transgene expression		
	Day 10	Day 28	Day 60
Control	17.55 $\pm$ 5.63	0	0
CD4 Ab	18.34 $\pm$ 7.31	12.36 $\pm$ 4.78	8.29 $\pm$ 2.18
CD40L Ab	15.52 $\pm$ 7.85	9.45 $\pm$ 4.46	6.12 $\pm$ 3.33
Cyclophosphamide	17.32 $\pm$ 4.96	18.06 $\pm$ 6.16	14.09 $\pm$ 6.14

Data were quantified with Quantimet 500+ (Leica) by analyzing the whole cross sections of tibialis anterior muscles from a total of six legs for the expression of lacZ at days 10, 28 and 60.

Data represent mean  $\pm$  SD.

#### DISCUSSION

The goal of this study was to develop a model that explains the immune responses to adenoviral vector delivered to skeletal muscle. Strategies for overcoming problematic host responses were developed based on our understanding of the basic cellular and molecular interactions involved.

A major limitation of E1 deleted vectors for the treatment of genetic diseases has been the short duration of expression and associated inflammation (13). Previous studies in liver and lung indicate that expression of neoantigens in the target cells (i.e., viral proteins and the transgene product) activate CD8 $^+$  T cells to form CTLs which directly or indirectly extinguish gene expression (11-14). It is clear that antigen specific immune responses cannot explain the entire spectra of associated inflammation. Furthermore, cellular immune responses are not the only factors involved in destabilizing transgene expression. Previous studies in mouse skeletal muscle confirmed the importance of cellular immunity in the performance of adenoviral vectors by demonstrating prolonged transgene expression in mice with global defects in immune function (8-10) as well as animals deficient in  $\beta_2m^-$  [i.e., void of MHC class I and CD8 $^+$  T cells (9)]. The demonstration in our studies of stabilized transgene expression in RAG-2 $^-$  and  $\beta_2m^-$  mice confirms the importance of CD8 $^+$  T cell effectors in this process. Vector administration was also associated with activation of B cells that secreted substantial quantities of antiviral neutralizing antibodies which diminish efficiency of gene transfer following repeated dosings.

The cellular immune responses to adenovirus infected skeletal muscle were characterized both *in vitro* and *in vivo*. Three sources of antigens were considered for activation of CD8 $^+$  T cells including viral capsid proteins, newly expressed viral proteins from open reading frames retained in the vector, and the transgene product  $\beta$ -galactosidase. *In vitro* chromium release assays confirmed that input capsid proteins do not activate detectable numbers of CTLs, presumably because exogenous proteins are not efficiently presented by MHC class I. CTL was detected *in vitro* to both  $\beta$ -galactosidase and newly expressed viral proteins which is similar to previous studies of adenoviral vectors delivered to liver and lung (23,24). Vilquin *et al.* have previously shown antibodies to  $\beta$ -galactosidase under similar conditions although they point out it is unlikely that these antibodies contribute to transgene instability (10). Experiments in the ROSA-26 mouse, where  $\beta$ -galactosidase is eliminated as an antigen, demonstrated stability of transgene expression despite the presence of CTLs to newly expressed viral proteins

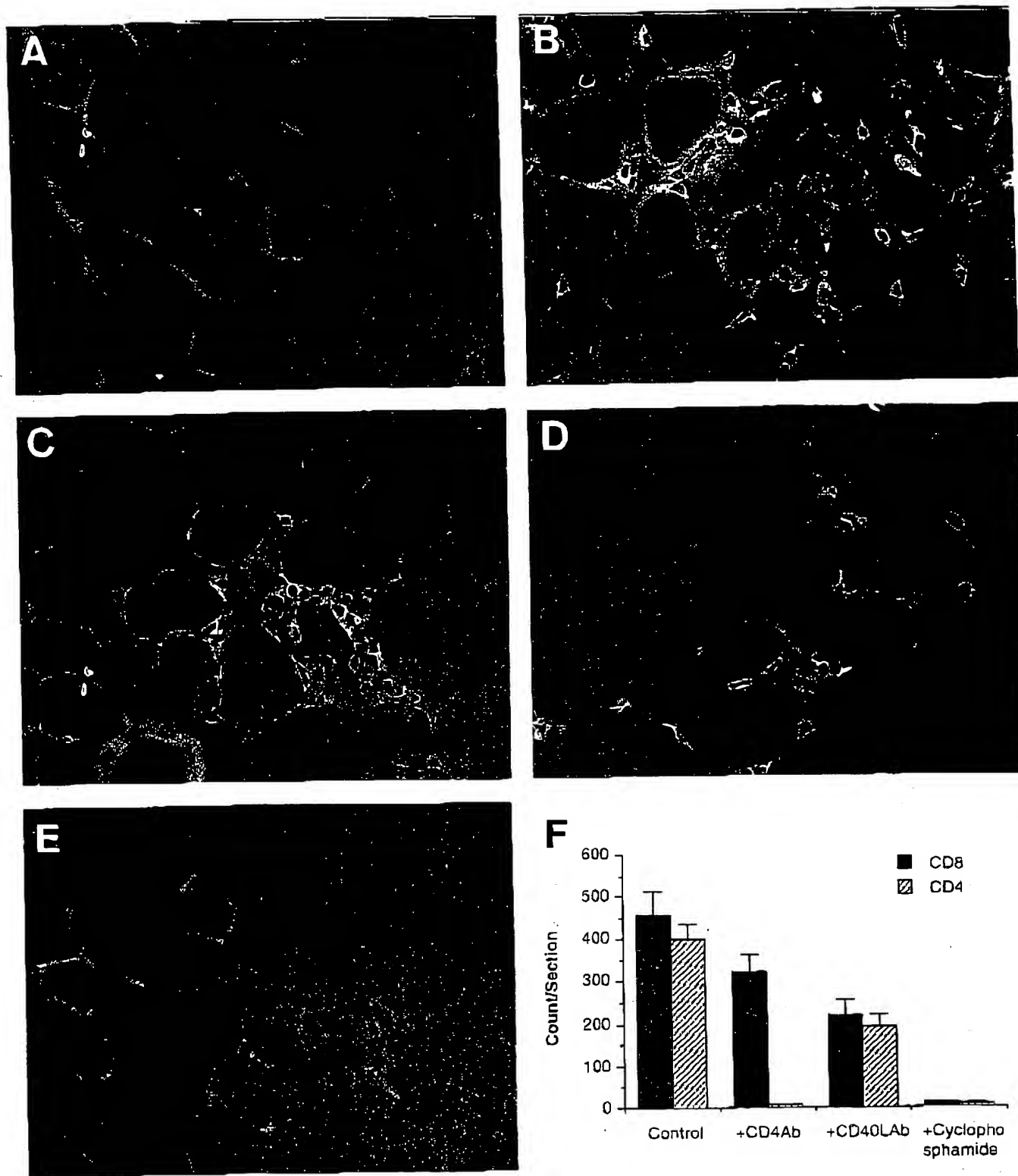
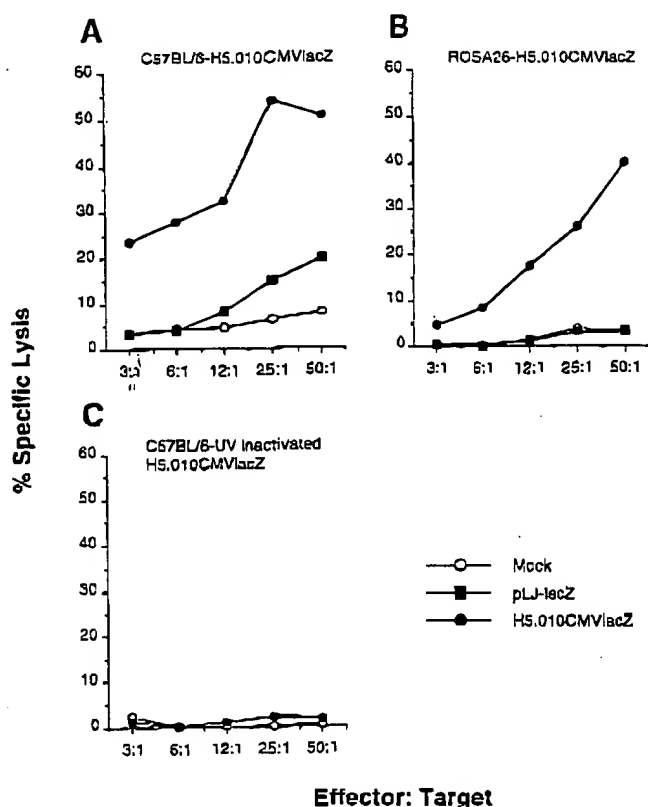


Figure 3. Infiltration of CD8<sup>+</sup> and/or CD4<sup>+</sup> lymphocytes in mouse muscles following adenovirus infusion. Frozen sections of muscles from naive C57BL/6 mice (A) and H5.010CMVlacZ-infected C57BL/6 mice at day 10 (B, C, D and E) were evaluated for the presence of CD4<sup>+</sup> and/or CD8<sup>+</sup> cells by double immunofluorescence. Shown are representative data derived from bilateral sections of three individual mice. (B) Control C57BL/6 mice; (C) mice treated with CD4 Ab; (D) mice treated with CD40L Ab; (E) mice treated with cyclophosphamide; (F) a summary of the quantitative analysis of CD4<sup>+</sup> and/or CD8<sup>+</sup> cell infiltrate. In all cases, infiltration of lymphocytes was substantially reduced on days 30 and 60. Magnification  $\times 400$ .





**Figure 4.** Activation of T cells in adenovirus-mediated gene transfer to mouse muscles. Lymphocytes harvested from mice 10 days after administration of vector were restimulated *in vitro* for 5 days, and tested for specific lysis on mock-infected ('Mock'), pLJ-lacZ-infected ('pLJ-lacZ') and H5.010CMVlacZ-infected ('H5.010CMVlacZ') C57SV cells in a 6 h  $^{51}\text{Cr}$  release assay. Percentage of specific lysis is expressed as a function of different effector to target ratios (3:1, 6:1, 12:1, 25:1 and 50:1). (A) C57BL/6 mice infected with H5.010CMV lacZ; (B) ROSA-26 mice infected with H5.010CMVlacZ; (C) C57BL/6 mice infected with UV inactivated H5.010CMVlacZ.

suggesting cellular immune responses to  $\beta$ -galactosidase dominate in muscle of non *lacZ* transgenic animals. This result should be considered in the context of previous studies of gene transfer to muscle using other vectors, as well as studies of adenovirus-mediated gene transfer to other tissues such as liver and lung.

Prior studies of gene transfer to skeletal muscle have yielded conflicting results regarding the immunogenicity of  $\beta$ -galactosidase. Hughes and Blau studied cell lineage in mouse skeletal muscle by transplanting primary myoblasts retrovirally marked with *lacZ*. They showed incorporation of the  $\beta$ -galactosidase expressing myoblasts into the muscle fibers and long term transgene expression (25). In other studies, direct injection of plasmid DNA containing a *lacZ* minigene into mouse skeletal muscle yielded substantial transfection *in vivo* based on X-gal histochemistry, which diminishes to undetectable levels by 2 months; this contrasts with identical experiments with a luciferase plasmid where transgene expression is prolonged (26). We conclude that  $\beta$ -galactosidase has the potential for eliciting destructive immune responses, however, this is highly dependent on the method of gene transfer. Direct gene transfer with plasmid or adenoviral vector may enhance the immune response by

infecting antigen presenting cells located within the injected muscle or in regional lymph nodes.

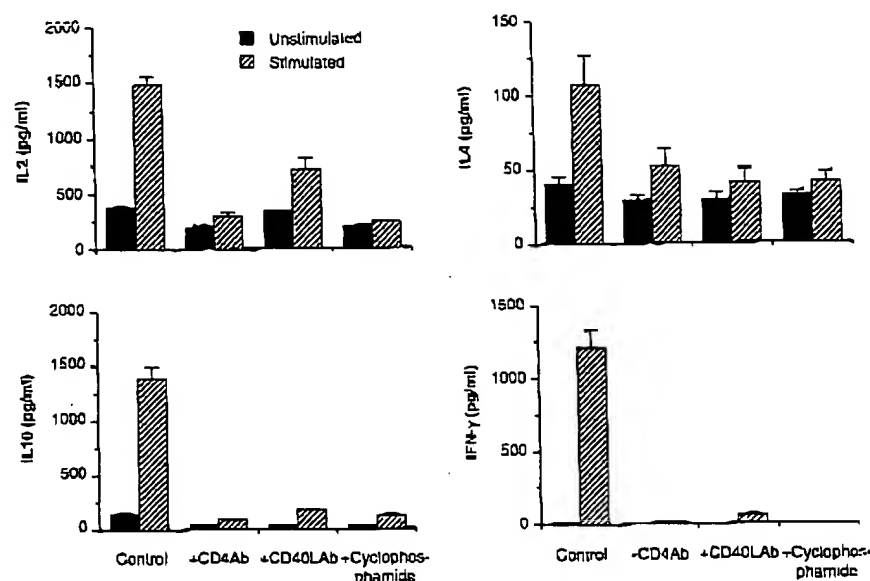
The importance of  $\beta$ -galactosidase to the cellular immune response in muscle differs from our experience with liver and lung where it has been shown that CTLs + viral antigens alone are sufficient to destroy transduced cells and extinguish expression (23,24). Our aggregate experience with adenoviral vectors in three different target tissues has raised several interesting issues. It is clear that analysis of CTL activity from bulk cultured lymphocytes that are restimulated *in vitro* is not quantitative and may not predict the relative importance of CTL populations for *in vivo* responses. The basic principles of immunology of *in vivo* gene therapy may differ qualitatively with respect to target tissue. Even more subtle differences in the experimental model such as dose and route of vector administration, age and strain of animal, and vector structure may play a role. The reason why muscle fibers harboring an E1 deleted adenoviral vector are not effectively targeted by antiviral CTLs is unclear. It is possible that CTLs are activated in regional lymph nodes by antigen presenting cells infected *in situ* by disseminated virus. The muscle fiber may express and/or present insufficient antigen to engage activated CTLs. We have in fact been unable to detect early (i.e., E2a) or late viral gene expression (i.e., hexon, penton or fiber) in transduced murine skeletal muscle. In light of these findings, it will be interesting to assess in models of skeletal muscle directed gene transfer the utility of second generation adenoviral vectors deleted of additional viral genes (27).

Our study demonstrated significant activation of  $\text{CD4}^+$  T cells to input viral capsid proteins. Characterization of the cytokine profile of T cells following stimulation with virus indicated both  $\text{T}_\text{H1}$  (i.e., modulate cellular immunity) and  $\text{T}_\text{H2}$  (i.e., modulate humoral immunity) subsets of T helper cells were activated. The functional importance of  $\text{T}_\text{H}$  cell activation was evaluated in C57BL/6 mice in which  $\text{CD4}^+$  T cell function was transiently inhibited by injecting either  $\text{CD4}$  depleting mAb or an mAb to  $\text{CD40L}$  that interfered with an important costimulatory pathway. Both mAbs blunted  $\text{CD4}^+$  T cell activation based on *in vitro* stimulation assays; the *in vivo* correlate was prolongation of transgene expression. Neutralizing antibody, which presumably requires thymus dependent B cell activation, was also diminished. Importantly, these direct and indirect indices of T cell function remained suppressed long after the function of the immune modulator is diminished. These data suggest a model similar to that seen in the liver and lung, i.e., that  $\text{CD4}^+$  T cell activation to input viral capsid proteins is necessary for effector responses of  $\text{CD8}^+$  T cells and B cells.

The practical implication of this study is that destructive humoral and cellular responses to adenoviral vectors in skeletal muscle may be prevented if  $\text{CD4}^+$  T cell activation is inhibited. A direct comparison of three immune modulators—cyclophosphamide,  $\text{CD4}$  mAb,  $\text{CD40L}$  mAb—indicated that cyclophosphamide consistently yielded the best result. Inflammation, including  $\text{CD4/CD8}$  infiltration, was prevented at day 10. Activation of  $\text{CD4}^+$  T cells based on *in vitro* studies was blunted and no neutralizing antibody was formed. Importantly, transgene expression was stable without diminution for at least 2 months.

The preferred approach for preventing destructive CTLs is to restructure the vector so that neoantigens are not expressed (13). This, however, may not be possible for replacement therapy of recessive diseases where the therapeutic protein is a potential neoantigen by definition. An example is the somatic replacement





**Figure 5.** The effect of immune modulators on cytokine secretion in adenovirus-mediated gene transfer to mouse muscles. Lymphocytes from C57BL/6 mice ('Control') 10 days after infection or mice treated with CD4 Ab ('+CD4 Ab'), CD40L Ab ('+CD40L Ab') or cyclophosphamide ('+Cyclophosphamide') were cultured with ('Stimulated') or without ('Unstimulated') UV-inactivated virus for 24 h, and the supernatants were assayed for the production of IL2, IL4, IL10 and IFN- $\gamma$  (pg/ml) by cytokine-specific ELISA.

of the LDL receptor gene with an adenoviral vector in the liver of a genetically deficient mouse model where immune responses to the therapeutic protein demonstrate the response of the host (28). Furthermore, restructuring the vector genome to eliminate neoantigen expression will not render the capsid proteins of the virion less immunogenic to B and T helper cells. It is probable, therefore, that a strategy of transient immune blockade with vector, such as that described in this study will be required.

## MATERIALS AND METHODS

### Administration of recombinant adenoviruses into mouse muscles

Control experiments were performed with C57BL/6 mice (H-2<sup>b</sup>) and 129 mice (H-2<sup>b</sup>) (obtained from Jackson Laboratory, Bar Harbor, Maine) with identical results. RAG-2<sup>-</sup> and  $\beta_2$ m<sup>-</sup> mice were purchased from GenPharm International (Mountain View, California). A description of the specific immune defects present in these mice is provided in the Results. RAG-2<sup>-</sup> mice are in 129 background (H-2<sup>b</sup>).  $\beta_2$ m<sup>-</sup> mice were bred onto a C57BL/6 background (>5 generations). ROSA-26 mice, purchased from Jackson Laboratories, are on a 129 background. For depletion of CD4<sup>+</sup> T cells or blocking of CD40L-CD40 interaction, mice were injected intraperitoneally with 0.5 ml aliquots of 1:10 dilution of mouse ascites fluid containing the GK1.5 (anti-CD4, ATCC) or MR1 (anti-CD40L, ATCC) mAb at days -1, 0, +3 and +6. This dose of ascites contained ~100  $\mu$ g of antibody. Some mice were treated intraperitoneally with 100 mg/kg of cyclophosphamide at days 0, +3 and +6.

Female mice (4–5 week-old) were selected for this study. The recombinant adenovirus H5.010CMVlacZ ( $5 \times 10^8$  p.f.u.) in 25  $\mu$ l of phosphate-buffered saline (PBS, pH 7.4) was directly injected into tibialis anterior muscles. This vector is deleted in E1, has a small substitution in E3, and expresses lacZ from a CMV

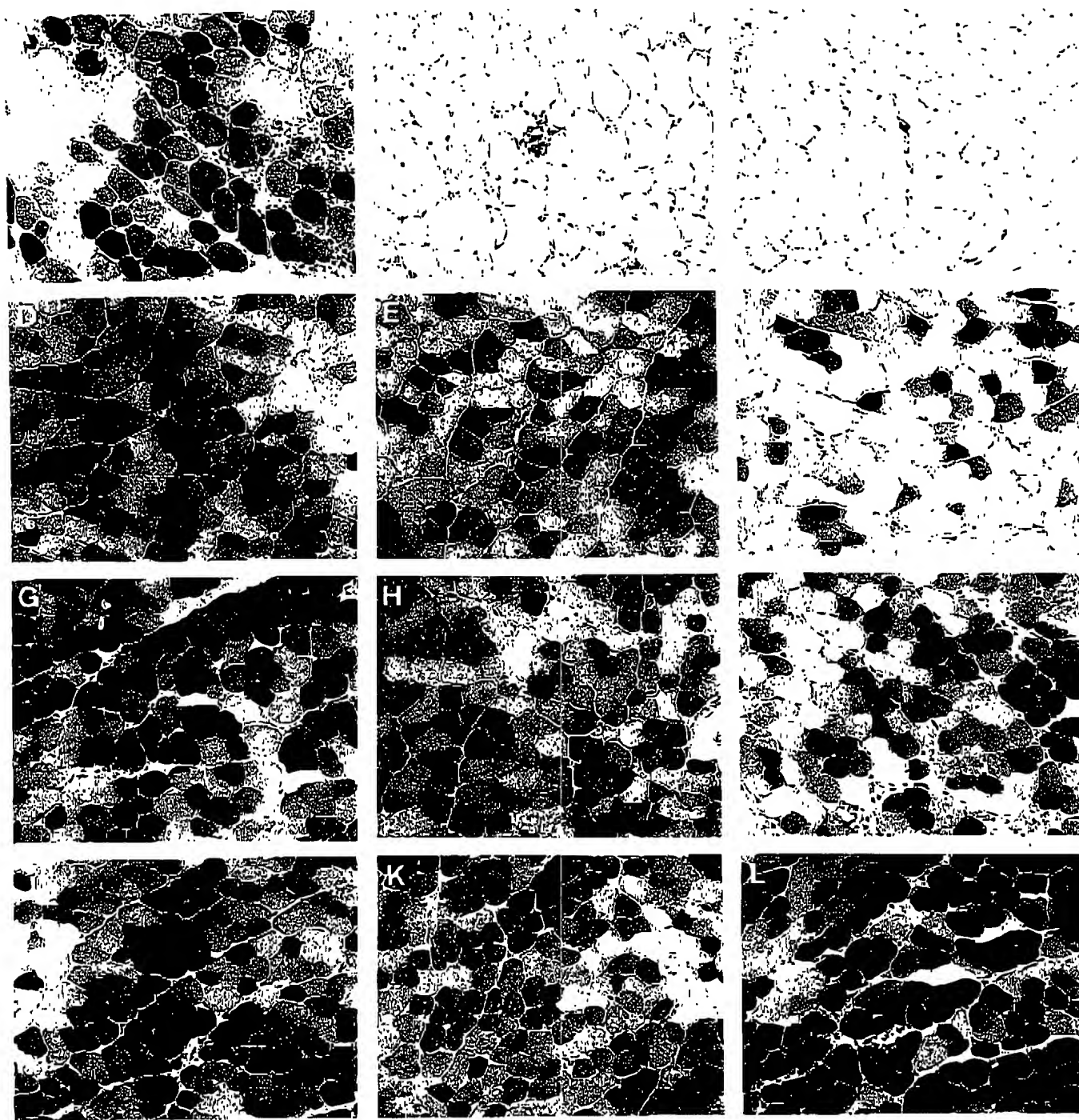
enhanced  $\beta$ -actin promoter. Selected stocks of virus are screened for presence of replication competent adenovirus (RCA); the current assays consistently demonstrate RCA at levels no greater than  $1:10^6$ . When animals were necropsied 10, 17, 28 or 60 days later, muscle tissues were snap-frozen in liquid nitrogen-cooled isopentane and sectioned at 6  $\mu$ m thickness, while serum samples and the draining inguinal lymph nodes were harvested for immunological assays. Analysis of tissue at each time point represented a minimum of 6 injection sites (i.e., bilateral sampling from at least three animals).

### Morphological analyses

**X-gal histochemistry.** Frozen sections (6  $\mu$ m) were fixed in 0.5% glutaraldehyde and stained for  $\beta$ -galactosidase activity as described. Sections were counterstained in neutral red solution.

**Detection of CD8<sup>+</sup> and CD4<sup>+</sup> cells by immunofluorescence.** Frozen sections (6  $\mu$ m) were fixed in methanol and stained with anti-CD4 and anti-CD8 antibodies as described. Morphometric analysis was performed to quantify the number of CD8<sup>+</sup> and CD4<sup>+</sup> cells per section.

**Pathology.** Sections (6  $\mu$ m) were fixed with formalin and stained with hematoxylin and eosin (H & E) according to standard procedures. Random sections in a blinded fashion were evaluated for histopathology using the criteria developed by Rostami for describing polymyositis with minor modifications (H.Nemoto, D.L.Schotland and A.Rostami, in preparation). Within each section, the severity of pathology was quantified based on a grade from 0 (no pathology) to 3 (severe pathology). Grade 0: no inflammation; grade 1: endomysial inflammation with a minimum of four muscle fibers undergoing necrosis and/or degeneration in the muscle cross section; grade 2: focal perimysial inflammation plus grade 1 changes; grade 3: diffuse perimysial

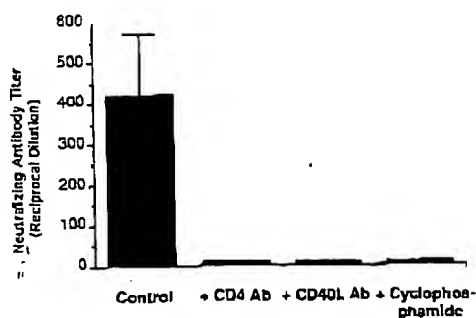


**Figure 6.** The effect of immune modulators on the stability of adenovirus-mediated transgene expression in muscles. H5.010CMVlacZ ( $5 \times 10^8$  p.f.u.) was injected intramuscularly into C57BL/6 mice (first row), C57BL/6 mice depleted of CD4<sup>+</sup> cells (second row), C57BL/6 mice treated with CD40L Ab (third row) and C57BL/6 mice treated with cyclophosphamide (fourth row). Mice were sacrificed and muscle tissues were harvested 10 (first column), 28 (second column) and 60 (third column) days later for lacZ expression by X-gal histochemistry. Magnification  $\times 200$ .

inflammation plus grade 1 changes. Focal inflammation represents one or two areas of inflammation while diffuse areas were greater than two inflammatory zones. Analyses were performed on three animals per time point encompassing two sections for each animal.

#### CTL assay

Lymphocytes harvested from inguinal lymph nodes were restimulated with H5.010CMVlacZ at moi of 1 for 5 days at  $6 \times 10^6$  cells/well (in 24 well plate) in 1.6 ml of Dulbecco's modified



**Figure 7.** Anti-viral neutralizing antibody. Serum samples obtained 28 days after infection from C57BL/6 mice ('Control'), C57BL/6 mice depleted of CD4<sup>+</sup> cells ('+CD4 Ab'), C57BL/6 mice treated with CD40L Ab ('+CD40L Ab') and C57BL/6 mice treated with cyclophosphamide ('+Cyclophosphamide') were tested for the presence of neutralizing antibodies to adenovirus. This figure summarizes neutralizing antibody titer present in serum samples.

Eagle's medium (DMEM) supplemented with 5% FBS and 50  $\mu$ M 2-mercaptoethanol. A standard 6 h  $^{51}$ Cr-release assay was performed subsequently using different ratios of effector to target cells (C57SV, H-2<sup>b</sup>) in 200  $\mu$ l DMEM in V-bottom 96-well plates. Prior to mixing with the effector cells, target cells were either infected with H5.010CMVlacZ or stably transduced with a lacZ-expressing retrovirus, pLJ-lacZ, labeled with 100  $\mu$ Ci  $^{51}$ Cr and used at  $5 \times 10^3$  cells/well. After incubation for 6 h, aliquots of 100  $\mu$ l supernatant were counted in a gamma counter. Percentage of specific  $^{51}$ Cr release was calculated as: [(c.p.m. of sample - c.p.m. of spontaneous release) / (c.p.m. of maximal release - c.p.m. of spontaneous release)]  $\times$  100. Spontaneous release was determined by culturing target cells in medium and maximal release was established by culturing target cells in a 1% solution of SDS.

### Cytokine release assay

Lymphocytes ( $6 \times 10^6$ ) were restimulated for 40 h with or without UV-inactivated H5.010CMVlacZ in 1.6 ml DMEM in 24-well Costar plates. Cell-free supernatants (100  $\mu$ l) were assayed for the secretion of IL2, IL4, IL10 and IFN- $\gamma$  by ELISA or transferred onto  $2 \times 10^3$  HT-2 cells (IL-2 and IL-4-dependent cells) or CT4S cells (IL-4-dependent cells) on round bottom 96-well plates. Proliferation was measured 72 h later by a 8 h [ $^3$ H]thymidine (0.50  $\mu$ Ci/well) pulse. Data reflect the mean of quadruplicate samples.

### Neutralizing antibody assay

Mouse serum samples were incubated at 56°C for 30 min to inactivate complement and then diluted in DMEM in 2-fold steps starting from 1:20. Each serum dilution (100  $\mu$ l) was mixed with H5.010CMVlacZ ( $1 \times 10^6$  p.f.u. in 20  $\mu$ l), incubated for 1 h at 37°C, and applied to 80% confluent HeLa cells in 96-well plates ( $2 \times 10^4$  cells/well). After 60 min incubation at 37°C, 100  $\mu$ l DMEM containing 20% FBS was added to each well. Cells were fixed and stained for  $\beta$ -galactosidase expression the following day. All of the cells stained blue in the absence of serum samples. The titer of neutralizing antibody for each sample was reported as the highest dilution with which <50% of cells stained blue.

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